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Overexpression of P-glycoprotein (Pgp), 170-kDa glycoprotein coded by the MDR1 gene in humans, confers multidrug resistance in various tumors. Pgp actively pumps various substrates including chemotherapeutic agents out of cells and is associated with swelling-activated chloride currents (I_{Cl}^s). Our aim is to determine the relationship between Pgp structure and function. We determined regulatory elements involved in C-half topogenesis. Our results indicate that C-half topogenesis is (1) independent of N-half sequence, (2) independent of expression system, and (3) partially regulated by charged amino acids surrounding transmembrane segments. To investigate the relationship between Pgp and I_{Cl}^s, we plan on characterizing the channel(s) underlying I_{Cl}^s in isolated rat hepatocytes and blood cells. Using the whole-cell patch clamp technique, we examined several properties of I_{Cl}^s in rat hepatocytes and observed (1) outwardly-rectifying currents, (2) minimal current inactivation at positive voltages, (3) I_{Cl}^s activation without pipette ATP, (4) block of with NPPB or DIDS, and (5) selectivity sequence of $SCN^- > I^- \cong NO_3^- \cong Br^- > Cl^- >$ gluconate. Our aim is to study the same properties in isolated rat blood cells to establish whether or not the channel accounting for I_{Cl}^s is the same.

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Ernest H 9/23/96

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Background and Significance

The treatment of breast and other cancers with chemotherapeutic agents often fails because cancer cells are either intrinsically resistant or develop resistance to multiple chemotherapeutic agents (termed multidrug resistance or MDR) (1,2). A major mechanism underlying MDR is the overexpression of the *MDR1* gene in humans which encodes a 1280 amino acid membrane glycoprotein called P-glycoprotein (Pgp). Pgp is thought to be a bifunctional protein that actively pumps out a diverse group of substrates and has been associated with volume-activated chloride currents (3,4). Recent studies have demonstrated multiple Pgp topologies in both endoplasmic reticulum and plasma membrane (5-11). However, little is known about how these Pgp structures relate to its functions. The goal of my research is to examine this relationship in order to understand the mechanisms involved in chemotherapy failure for the treatment of breast cancer and other malignancies.

I. Structure of P-glycoprotein

P-Glycoprotein is a member of the ABC (ATP-binding cassette) superfamily of transporters which also includes the cystic fibrosis transmembrane conductance regulator (1,2). Based on hydropathy analysis, most of the ABC superfamily members consist of two homologous domains, each consisting of six transmembrane segments followed by a nucleotide-binding region. However, topological analysis of N- and C-terminal half Pgp has demonstrated alternate topologies in both the plasma membrane and endoplasmic reticulum (5-11). In my previous annual research report (for the period Sept.1, 1994- Aug. 31, 1995), we demonstrated an alternate topology for the C-terminal half of Chinese hamster *pgp1* Pgp using a cell-free expression system. We proposed that transmembrane segments (TM) 7 and 9 span the membrane while TM8 is in the lumen (manuscript in preparation). Other models have been generated based on topological studies using both different expression systems and Pgp species (6,9-10,12-14). Although there is disagreement over Pgp topology, an interesting question is how the generation of the various Pgp topologies is regulated. Recent studies of the regulation of N-terminal half Pgp topogenesis (15-17) reported that (1) charged amino acids surrounding TM segments can determine membrane orientation, but not targeting to ER membranes, (2) cytoplasmic factors present in the wheat germ translation system can modulate topology, and (3) temperature can alter the membrane orientation and insertion process co-translationally. For the C-terminal half of Pgp, little is known about the regulatory mechanisms of topogenesis. Here, we report the biogenesis of C-terminal half Pgp, considering (1) the role of the N-terminal half Pgp sequence, (2) the effect of cytoplasmic factors in the wheat germ translation system, and (3) the effect of charged amino acids surrounding TM segments. Based on these results, we propose to "lock" Pgp in one topology and assess its function in intact cells.

II. Function of P-glycoprotein

Pgp has been shown to actively extrude a wide variety of substrates out of cells (1,2).

which includes chemotherapeutic agents, peptides and steroids. In addition, Valverde *et al.* (3) reported that Pgp was associated with volume-activated chloride currents in NIH3T3 mouse fibroblast or lung epithelial cell lines transfected with the human *MDR1* Pgp cDNA. However, several other investigators have been unable to confirm this result (18-24). We recently have demonstrated a specific role for Pgp and swelling-activated chloride currents (I_{Cl}^s) (25). Under whole-cell patch clamp conditions, the addition of an anti-Pgp monoclonal antibody (mAb) C219 to the pipette solution blocked I_{Cl}^s in a human breast cancer cell line transfected with human *MDR1* cDNA. Control IgG or another anti-Pgp monoclonal antibody (JSB-1) had no effect. The C219 effect was also demonstrated in other cells expressing functional Pgp in the plasma membrane (KB-V1 cells). Thus Pgp may represent a bifunctional protein, which transports a diverse group of substrates and is either itself a chloride channel or a regulator of endogenous Cl^- channels.

To facilitate interpretation of future studies involving Pgp and I_{Cl}^s, we characterized in detail the properties of I_{Cl}^s. Although the molecular identity of a few chloride channels has been determined, the DNA or amino-acid sequence encoding volume-activated chloride channel(s) has remained elusive. Based on studies to date, essentially all mammalian cells possess an outwardly-rectifying anion conductance activated by cell swelling (26). These conductances have been characterized in numerous cell types and found to be similar. However, upon closer inspection certain variations do exist (e.g., current inactivation at positive voltages). Recently, Strange *et al.* (26) proposed that although variations in conductance properties have been reported between various cell types, a single channel type could still account for the conductance found in various cells. Point mutations present in the channel or differences in methods of current analysis may explain rather small variations.

Significant differences in I_{Cl}^s were observed between two Pgp-expressing cell lines, mouse fibroblast cell line (BALB/c-3T3) transfected with Chinese hamster *pgp1* Pgp cDNA and a MDR Chinese hamster lung fibroblast cell line (LZ-8) (Vanoye, Altenberg, Reuss; manuscript in preparation). Both cell lines display a C219-sensitive I_{Cl}^s, but differ significantly in (1) chloride channel blocker sensitivity, (2) ionic selectivity, and (3) ATP requirement for current activation. It would be difficult to ascribe currents with such diversity of properties to a single channel type. However we cannot rule out the possibility of small species-related differences in amino acid sequence of the channel(s) involved. Since it has yet to be determined whether Pgp is a chloride channel itself or a regulator of endogenous channels, we investigated the detailed properties of I_{Cl}^s (such as ATP dependence for activation, anion selectivity). Our aim is to characterize and compare I_{Cl}^s between isolated rat hepatocytes and blood cells in order to gain insight into the channel or channels underlying I_{Cl}^s. These studies will facilitate our interpretation of future studies involving functional evaluation (esp. I_{Cl}^s) of cells expressing topological mutants of Pgp.

BODY OF ANNUAL REPORT

METHODS

I. Regulation of C-terminal half Pgp topogenesis at the endoplasmic reticulum

I.1 *cDNA constructs and site-directed mutagenesis*

cDNA fragments encoding either the full-length or the six transmembrane segments of the C-terminal half of hamster *pgp1* Pgp were subcloned into a pGEM-4z expression vector to generate pGHaPGP-F and pGHaPGP-C6, respectively. For experiments involving the addition of charged amino acids to the C-terminal half sequence, site-directed mutagenesis in M13 was used as described previously (8). Briefly, pGHaPGP-C6 was subcloned into M13mp19 to generate pMHaPGP-C6. M13 DNA was propagated for three cycles in BW313 and the U-containing single strand DNA was prepared for synthesis of mutagenic strand. Restriction mapping of mutation-introduced restriction site was used to screen for mutant DNA. Following identification of the mutation, double-stranded M13 DNA was prepared by standard procedures. The mutated cDNA fragment was then subcloned into a pGEM-4z vector by ligating a fragment of pMHaPGP-C6 carrying the mutation with a fragment of pGHaPGP-C6 and a fresh pGEM-4z vector. Oligonucleotides used to generate the mutations were 5'- AGCTGGACGGATCCTCA-3' (E779R) and 5'- TTGGCAAACGTCGACGAGCTGGAGAG- 3' (RRR). E779R mutation represents the replacement of glutamic acid 799 with arginine. RRR inserted three additional arginines after lysine 776. DNA sequencing was performed for all DNA constructs to insure proper linkages during subcloning and the generation of desired mutations.

I.2 *In vitro transcription and translation using a cell free system*

Wild-type and mutant cDNA templates were linearized with a restriction enzyme (see Fig. 1B) and transcribed with SP6 RNA polymerase in the presence of a cap analog $m^7G(5')ppp(5')G$. Following transcription, the DNA template was removed by RQ1 DNase and RNA transcripts were purified by standard methods.

Translation of RNA transcripts was performed using the rabbit reticulocyte lysate (RRL) translation system in the presence of dog pancreatic microsomal membranes (RM) as suggested by the supplier (Promega). Translation products were centrifuged at 4°C and pellet fractions were isolated and resuspended in STBS solution (in mM: 250 sucrose, 10 Tris-HCl, pH 7.5, 150 NaCl) for further processing. For experiments requiring protease digestion and endoglycosidase treatment of membrane fractions, samples were exposed to 0.1-0.2 mg/ml proteinase K for 20 min at room temperature. After addition of PMSF (10 mM final concentration) to stop the reaction, the translation material was pelleted and washed with STBS solution containing PMSF. The pellet was resuspended in a reaction mix containing the following: (in mM) 50 sodium phosphate buffer (pH 7.6), 1.25% NP-40, 0.5% 2-mercaptoethanol, 2 PMSF, 1 unit PNGase F (or equivalent volume of water for control samples), and 0.2% SDS. After incubation for at least 1 hr, electrophoresis buffer was added and the sample was analyzed by SDS-PAGE and fluorography.

II. Functional analysis of swelling-activated chloride currents in isolated rat hepatocytes by whole-cell patch clamp technique

II.1 *Cells*

Either freshly isolated rat hepatocytes or hepatocytes maintained in UW medium for 1 day at 4°C were obtained for electrophysiological experiments. Cells were plated on a coverslip in L-15 medium at 37°C room air conditions 1-2 hrs prior to the experiment.

II.2 *Electrophysiology*

Whole-cell patch clamp configuration was achieved by obtaining gigaohm seals (using 4-7 MΩ resistance pipettes) and application of additional negative pressure and/or negative voltage to rupture the seal. Steady-state currents were measured at various voltages (-80 to +80 in 20 mV steps), 40 ms after the start of the voltage pulse. Bath and pipette solution compositions were as follows (in mM): NMDG-Cl pipette (280 mosmol/kg) - 140 N-methyl-D-glucamine chloride (NMDGCl), 1.2 $MgCl_2$, 1 EGTA, 10 HEPES, 2 ATP; Bath Isosmotic (280 mosmol/kg)- 140 NMDGCL, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 8 glucose; 11% Bath hyposmotic (250 mosmol/kg)- 122.5 NMDGCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 8 glucose; 22% Bath hyposmotic (220 osmol/kg)- 105 NMDGCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 8 glucose. For anion selectivity experiments, the bath solution was changed to an 11% hyposmotic NaCl bath solution (122.5 NaCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 8 glucose). Permeability to various anions were tested by changing to a bath solution consisting of 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 8 glucose and 122.5 NaX, where X = Br^-, I^-, NO_3^-, SCN^- or gluconate. All solutions were titrated to pH 7.4 and filtered.

RESULTS

I. Regulation of C-terminal half Pgp topogenesis

I.1 *Alternate C-terminal half topology is generated in the presence of N-terminal half hamster pgp1 Pgp.*

To determine whether the N-terminal half Pgp sequences are important in regulating C-terminal half topogenesis, we used mRNA transcripts encoding a truncated full-length hamster *pgp1* Pgp sequence (pGHaPGP-F/*XbaI* or pGHaPGP-F/*BclI*; see Fig. 1B) to direct translation using the rabbit reticulocyte lysate translation system in the presence of microsomal membranes (RM).

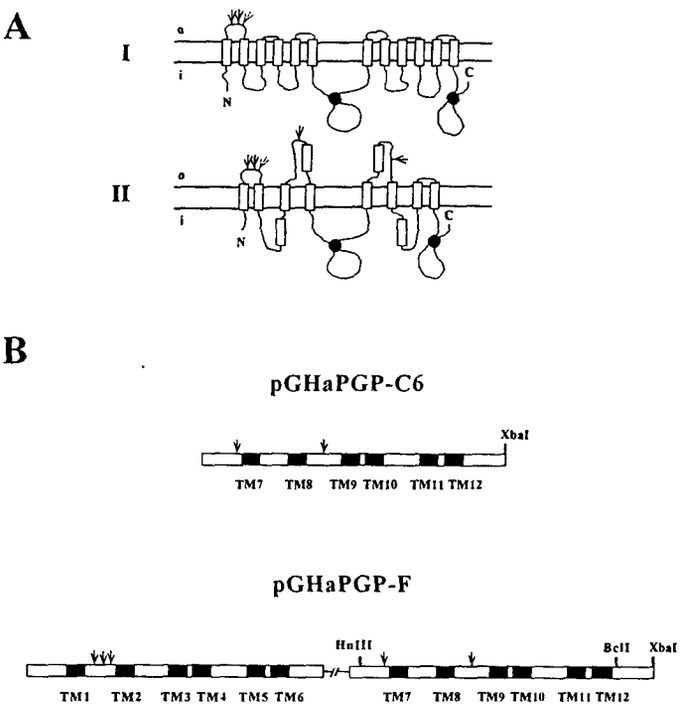


Figure 1. (A) Topological models of Pgp. Model I represents a topological structure based on hydropathy analysis of Pgp protein sequence (2). Model II was derived from topological studies of mouse *mdr1* (28) and hamster *pgp1* Pgp (7; see annual report 1994-1995). The symbol, i, represents cytoplasmic space while o is the extracellular space. Putative transmembrane domains are represented as rectangular boxes, and nonhydrophobic regions are designated as solid lines. (B) Schematic diagram of RNA transcripts encoding hamster *pgp1* C-terminal half (pGHaPGP-C6) or full-length (pGHaPGP-F) Pgp molecules. Restriction enzymes used to linearize cDNA constructs are indicated above the transcripts (see methods). Putative transmembrane regions are represented by solid bars and nonhydrophobic sequences are shown as open bars. Putative glycosylation sequences are denoted by branched symbols.

When the translation products associated with RM were digested with proteinase K, 17- and 19-kDa protease-resistant peptides were observed (Fig. 2A, lanes 1 and 3). Upon further treatment with the endoglycosidase, PNGase F, the bands shifted to 11- and 14 kDa suggesting that the 17- and 19- kDa bands were glycosylated (Fig. 2A, lanes 2 and 4).

(unpublished data)

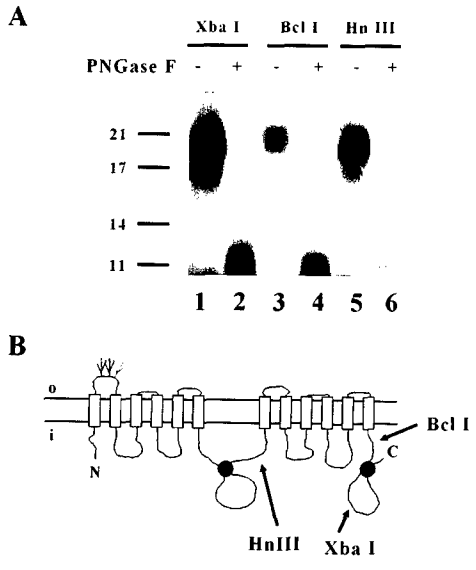


Figure 2. (A) Proteinase K digestion of microsomal membranes containing various truncated full-length Pgp molecules (lanes 1,3,and 5). PNGase F treatment, which removes core glycosylated oligosaccharides, resulted in a shift in molecular weight (lanes 2,4, and 6). (B) Schematic representation of Pgp topological model and the various truncation sites generated after linearization of cDNA with a restriction enzyme. Digestion with *HnIII* results in a N-terminal half Pgp molecule whereas *BclI* or *XbaI* include both the N-and C-terminal halves.

Three potential glycosylation sites are present within the first extracytoplasmic loop and core glycosylation of all three sites would correspond to an $\approx$8 kDa change. Thus it is likely that the 19-kDa band represents a glycosylated peptide fragment consisting of the amino acids from TM1 to TM2. The 17-kDa band is consistent with a glycosylated peptide fragment from the C-terminal half containing the amino acids from TM7 to 9, which was reported previously (see 1994-1995 annual fellowship report). To confirm this, we translated a RNA transcript encoding only the N-terminal half of Pgp. We noted a 19-kDa band, which shifted to 11 kDa upon exposure to PNGase F (Fig. 2A, lanes 5 and 6). However, we failed to observe both the 17- and 14- kDa products in either conditions. These results suggest that the C-terminal topology is not affected by the presence of N-terminal half sequences. The information which directs C-terminal half topogenesis is likely encoded within the C-terminal half itself.

I.2 *Cytoplasmic regulatory factors for the N-terminal half Pgp do not regulate C-terminal half topology.*

The topogenesis of the N-half Pgp was shown to differ in wheat germ extract (WGE) and rabbit reticulocyte lysate translation systems (RRL) (16). Cytoplasmic factors in WGE were identified and characterized as a heat-labile, soluble factor, which decreases the generation of N-terminal half alternate Pgp topology. To determine whether these factors played a role for regulation of C-terminal half topogenesis, translation was performed using premixed ratios of WGE and RRL. The ratios varied from complete RRL mixture to complete WGE mixture. pGHaPGP-C6/*XbaI* transcript was then added to initiate translation. Figure 3 shows that increasing the proportions of WGE to RRL did not decrease the generation of the glycosylated product (53-kDa band).

(unpublished data)

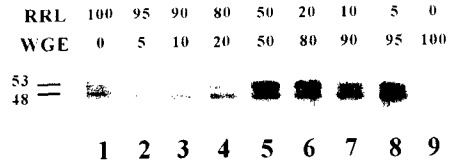


Figure 3. Wheat germ extract fractions do not affect generation of C-terminal half Pgp topology. Various amounts of RRL and WGE were premixed and pGHaPGP-C6/*XbaI* transcript was used to initiate translation. The percent fraction of RRL and WGE in each translation are indicated above the corresponding lane.

The 53-kDa band represents an alternate C-terminal half topology where the glycosylated loop between TM8 and 9 is present within the ER lumen (see 1994-1995 annual report). Zhang and Ling (16) had previously demonstrated that increasing the proportions of WGE to RRL resulted in the generation of a single, conventional N-half Pgp topology (see Figure 1A, model I). Our results suggest that the cytoplasmic factor(s) present in WGE regulates Pgp N-half but not C-half topogenesis.

I.3 *Addition of positively-charged amino acids behind putative TM8 decreases generation of C-terminal half alternate topology.*

Discrete regions called stop transfer (ST) sequences have been shown to mediate integration of proteins into membranes (27). ST sequences generally consist of a core hydrophobic region followed by a domain containing a net positive charge. These positive charges located in the cytoplasmic region may act as a "retention signal" for TM segments of polytopic membrane proteins. Based on previous results (see annual report 1994-1995), we generated a topological model for the C-terminal half hamster Pgp where TM7 and TM9 span the membrane while TM8 is present in the lumen of the ER. This model suggests that TM8 contains a modified amino acid sequence or lacks the proper information which codes for stop translocation and integration activity. To determine whether charged amino acids around the TM8 regulate C-half topogenesis, we modified the charge distribution immediately after TM8 by either (1) replacing glutamic acid residue 779 with arginine (E779R) or (2) adding three arginines behind lysine residue 776 (RRR) (Fig. 4B).

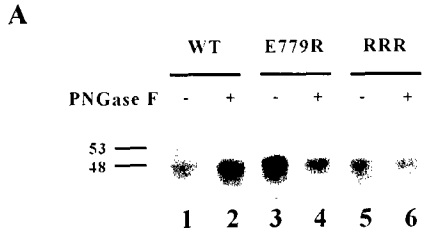
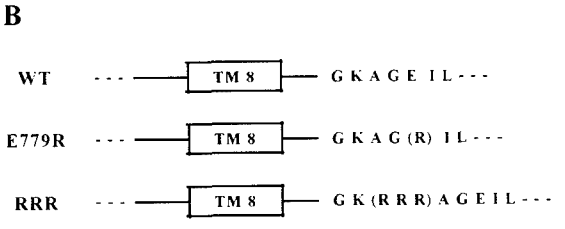


Figure 4. Membrane orientation of C-terminal half hamster Pgp with mutations C-terminal to TM8. (A) Membrane-associated fraction of the translation products WT (lanes 1-2),

(unpublished data)



E779R (lanes 3-4), and RRR (lane 5-6). (B) Schematic representation of the partial amino acid sequence encoding by wild-type, E779R, and RRR transcripts. Parentheses denote mutations created in the sequence.

Translation of a RNA transcript encoding E779R or RRR resulted in decreased ratio of the 53-kDa to 48-kDa band (Fig. 4A). This suggests that the generation of an alternate topology is decreased upon addition of positive charges following TM8. However it is noteworthy that even with the addition of three positive charges, TM8 could not completely integrate into membrane. Other mechanisms in addition to charge surrounding TM segments are likely to regulate the topogenesis of C-terminal half Pgp.

II. Characterization of swelling-activated chloride currents in isolated rat hepatocytes

II.1 *ATP dependence of current activation by cell swelling*

In this report we describe I_{Cl}^{s} in isolated rat hepatocytes using the whole-cell patch clamp technique. The hepatocytes displayed low basal currents under isosmotic bath conditions (Fig. 5B). Upon changing to hyposmotic bath solution for 8-10 min, an increase in current was observed (Fig. 5B).

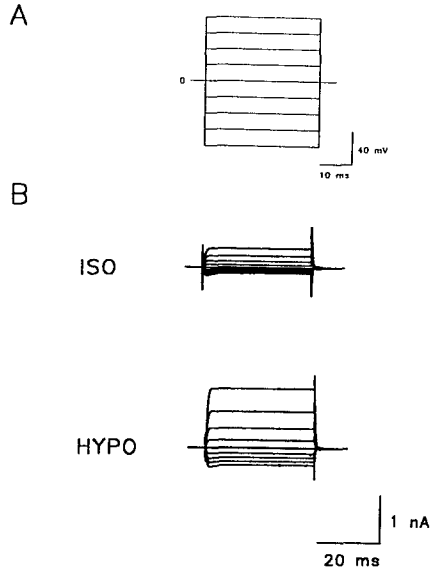


Figure 5. (A) Voltage pulse protocol. The pulses were given in 20 mV steps from -80 mV to +80 mV for 40 msec (holding potential = 0 mV). Whole-cell currents were measured at 40 msec of the onset of the voltage pulses (B) Typical whole-cell current records from isolated rat hepatocytes obtained with the voltage pulse protocol described in (A) during exposure to isosmotic or 11% hyposmotic bath conditions. Currents in hyposmotic solution were reversible (data not shown).

(unpublished data)

We noted that this current (1) was outwardly-rectifying with similar Cl^- concentrations on both sides of the cell membrane, (2) displayed slight (< 25%) inactivation of current at high positive voltages after 0.5 sec (data not shown), and (3) was highly selective for Cl^- over gluconate (permeability ratio $P_{Gluconate}/P_{Cl} < 0.1$; see below). Cl^- current activation by cell swelling requires intracellular ATP (26). Figure 6 shows however that removal of ATP from the pipette solution along with sufficient time (10 min) for dialysis of the intracellular compartment had no effect on activation of I_{Cl}^s.

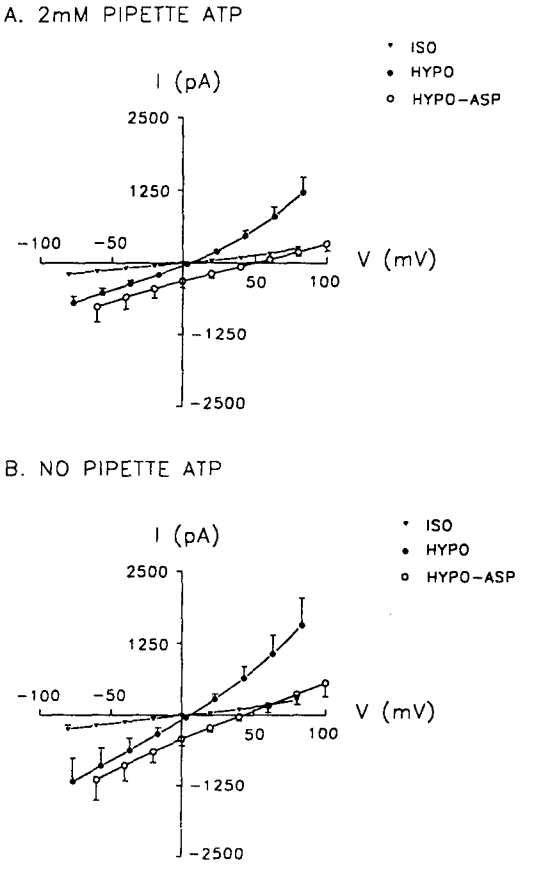


Figure 6. Steady-state current-voltage (I-V) relationship of I_{Cl}^s in the presence (A) or absence (B) of 2 mM ATP in the pipette solution. After obtaining whole-cell configuration, cells were bathed in isosmotic bath solution for 10 min (ISO) followed by a 10 min exposure to 22% hyposmotic bath solution (HYPO). Bath solution was then changed to 22% hyposmotic aspartic acid solution for 1-2 min (HYPO-ASP). Steady-states currents were measured at the end of each incubation period.

We noted that the success rate of maintaining whole-cell configuration was much lower (6 successful out of 20 experiment) when ATP was not included in the pipette solution in comparison to experiments where ATP was added to the pipette (8 out of 9 experiments). Intact whole-cell configuration was assessed by determining the Cl^- selectivity of the currents. A reversal potential change indicative of Cl^- current was observed upon replacing bath Cl^- in the hyposmotic solution with aspartate (Fig. 6). Taken together, the results suggest that ATP is not required for activation of I_{Cl}^s. However further experiments are necessary to ensure complete removal of cellular ATP.

(unpublished data)

II.2 *Chloride channel blockers, NPPB and DIDS, block* I_{Cl}^{s}

To further characterize I_{Cl}^{s} in rat hepatocytes, we tested the effects of the chloride channel blockers, NPPB and DIDS. After activation of I_{Cl}^{s} under hyposmotic bath conditions, the bath solution was changed to a hyposmotic bath solution containing either 0.1 mM NPPB or 1 mM DIDS for 8-15 min or 4 min, respectively. Both blockers significantly reduced the outward current (Fig. 7). The effects of both blockers were reversible upon returning to hyposmotic bath solution.

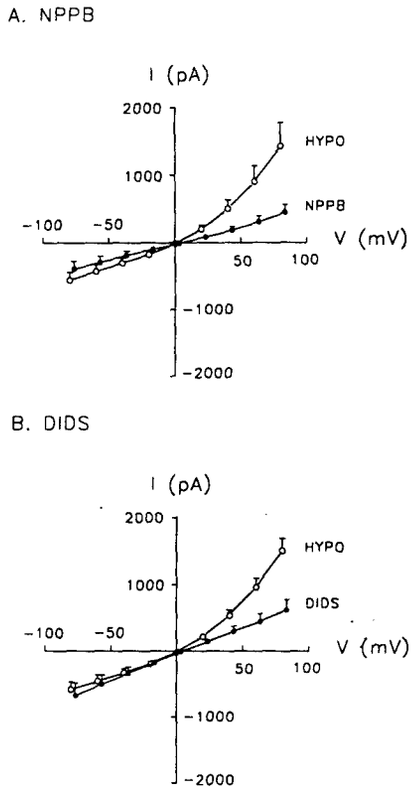


Figure 7. Steady-state current-voltage relationships in the presence of (A) 0.1 mM NPPB or (B) 1 mM DIDS. HYPO represents steady-state currents measured after 8-15 min exposure to hyposmotic bath solution. Bath solution was then changed to hyposmotic solution containing the blockers and currents were measured after 8-15 min or 4 min exposure to NPPB or DIDS, respectively. The blocker effect was reversible upon returning to hyposmotic bath solution.

II.3 *Determination of anion selectivity of* I_{Cl}^{s}

The anion selectivity sequence was determined for I_{Cl}^{s} by replacing the bath solution with various sodium salts of anionic compounds. The liquid junction potential generated between the reference electrode and bath solution was corrected by using the generalized Henderson equation. Ionic permeability ratios for the various anions tested were calculated from the change in reversal potential using the Goldman-Hodgkin-Katz voltage equation. The relative permeability ratios are presented in figure 8. Using one way ANOVA statistics, the relative permeability ratio for SCN^- was significantly larger than I^-, NO_3^-, and Br^-. However the permeability ratios for I^-, NO_3^- and Br^- were not statistically different from each other but were all greater than Cl^-. Gluconate permeability ratio was significantly smaller as compared to Cl^-. The permeability sequence is $SCN^- > I^- \cong NO_3^- \cong Br^- > Cl^- >$ Gluconate.

(unpublished data)

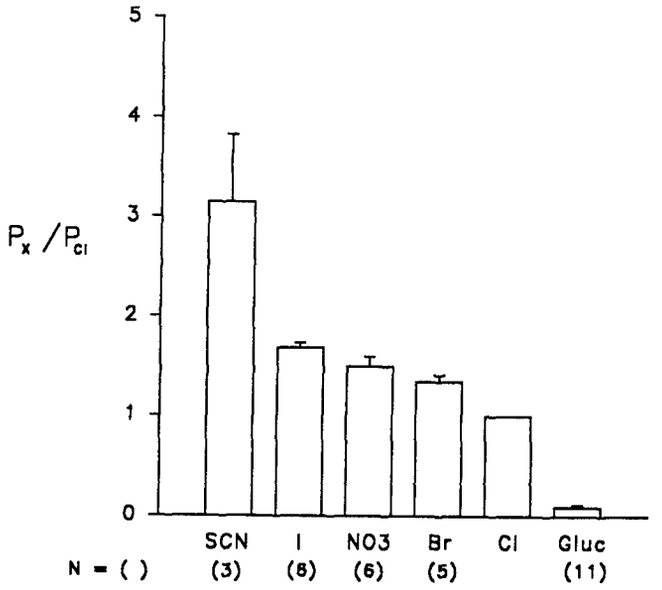


Figure 8. Ion selectivity of swelling-activated currents from isolated rat hepatocytes. P_x/P_{Cl} represents the permeability ratio of ion X with respect to Cl^-. Parentheses denote number of experiments for each anion tested. Reversal potential changes were measured after replacing NaCl 11% hyposmotic bath solution with a sodium salt of the following anions: SCN^-, I^-, NO_3^-, Br^-, gluconate (Gluc) (see Methods).

(unpublished data)

CONCLUSIONS

The aim of this project is to understand how the structure of Pgp relates to its functions. In this report we discuss experiments involved in "locking" Pgp into one topological orientation and assessed in detail the properties of I_{Cl}^s. We determined regulatory elements involved in the topogenesis of C-terminal half hamster *pgp1* Pgp, and showed that: (1) the N-terminal half Pgp sequences does not regulate the topogenesis of the C-terminal half molecule (2) C-terminal half topogenesis is independent of the expression system used and (3) charged amino acids flanking the C-terminal end of TM8 can modulate C-terminal half topology. The implications of these results are related to our proposal of "locking" Pgp into one topology and assessing its function. Although charged amino acids flanking TM segments did not result in the absolute generation of a single topological structure, there may be sufficient generation of one structure to assess its function in intact cells. A correlation between either I_{Cl}^s or drug transport and Pgp topology may be possible with expression of charge mutants.

To facilitate analysis of Pgp expression and I_{Cl}^s, we characterized various properties of I_{Cl}^s in isolated rat hepatocytes using the whole-cell patch clamp technique. We found that (1) outwardly-rectifying chloride selective currents were activated upon cell swelling, (2) activation of the current did not require intracellular ATP (3) the currents displayed little inactivation at highly positive voltages, (4) DIDS and NPPB blocked I_{Cl}^s, and (5) anion currents displayed the selectivity sequence $SCN^- > I^- \cong NO_3^- \cong Br^- > Cl^- >$ Gluconate. We plan to characterize I_{Cl}^s in isolated rat blood cells and compare them to results obtained in this report. By doing so we compare currents under similar electrophysiological conditions and avoid species-related differences in amino acid sequences encoding the channel(s) underlying I_{Cl}^s. Understanding the channel(s) involved in I_{Cl}^s will help us to define the relationship between I_{Cl}^s and wild-type or topological mutants of Pgp.

(unpublished data)

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